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Functioning and Nonfunctioning Thyroid Adenomas Involve Different Molecular Pathogenetic Mechanisms*

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ABSTRACT

The molecular biology of follicular cell growth in thyroid nodules is still poorly understood. Because gain-of-function (activating) mutations of the thyroid-stimulating hormone receptor (TShR) and/or Gsα genes may confer TSh-independent growth advantage to neoplastic thyroid cells, we searched for somatic mutations of these genes in a series of hyperfunctioning and nonfunctioning follicular thyroid adenomas specifically selected for their homogeneous gross anatomy (single nodule in an otherwise normal thyroid gland). TShR gene mutations were identified by direct sequencing of exons 9 and 10 of the TShR gene in genomic DNA obtained from surgical specimens. Codons 201 and 227 of the Gsα gene were also analyzed. At histology, all hyperfunctioning nodules and 13 of 15 nonfunctioning nodules were diagnosed as follicular adenomas. Two nonfunctioning thyroid nodules, although showing a prevalent microfollicular pattern of growth, had histological features indicating malignant transformation (a minimally invasive follicular carcinoma and a focal papillary carcinoma). Activating mutations of the TShR gene were found in 12 of 15 hyperfunctioning follicular thyroid adenomas. In one hyperfunctioning adenoma, which was negative for TShR mutations, a mutation in codon 227 of the Gsα gene was identified. At variance with hyperfunctioning thyroid adenomas, no mutation of the TShR or Gsα genes was detected in nonfunctioning thyroid nodules. In conclusion, our findings clearly define a different molecular pathogenetic mechanism in hyperfunctioning and nonfunctioning follicular thyroid adenomas. Activation of the cAMP cascade, which leads to proliferation but maintains differentiation of follicular thyroid cells, typically occurs in hyperfunctioning thyroid adenomas. Oncogenes other than the TShR and Gsα genes are probably involved in nonfunctioning follicular adenomas. (*J Clin Endocrinol Metab* **84:** 4155–4158, 1999)

THYROID NODULES are the most common thyroid disease, their prevalence being 4–7% in iodine-sufficient areas and much higher in iodine-deficient countries. Thyroid nodules are broadly subdivided in benign and malignant lesions (1). The molecular biology and cellular pathophysiology of thyroid cell growth in nodular lesions are still poorly understood (2, 3).

Pituitary thyroid-stimulating hormone (TSh) is the main stimulator of growth and function of normal follicular thyroid cells (2). TSh after binding to its membrane receptor (TShR) activates guanine-nucleotide-binding proteins Gs and Gq and stimulates the adenylate cyclase (AC)-cAMP pathway and the phospholipase C-diacylglycerol regulatory cascade, respectively (2, 4). Gain-of-function (activating) mutations have been detected in up to 80% of toxic adenomas in TShR gene and in up to 25% in the Gsα gene, suggesting that these genetic anomalies may play a role in the pathogenesis of hyperfunctioning nodules (5). However, discrepancy still exists on the frequency of TShR mutations in hyperfunctioning thyroid nodules (5). The lower prevalence observed in Japan, the United States, and in some Italian series (6) leaves room for other pathophysiological mechanisms or gene targets (5). In particular, studies in naturally occurring toxic adenomas suggested that constitutive activation of the Gsα protein-AC pathway may not be sufficient to generate these benign tumors (7).

Activation of the cAMP pathway, although not directly linked to malignant transformation, may concur in the development of malignant neoplasms, as shown by experimental animal models (8–10). Constitutive activation of the AC-cAMP pathway in transgenic mice expressing the A2 adenosine receptor in thyroid cells (8) results in the development of goiter and hyperthyroidism. Transgenic mice expressing the E7 protein of the human papillomavirus type 16, which inactivates the retinoblastoma gene product (Rb1) in the thyroid, develop simple goiter (9). Transgenic mice coexpressing the two oncogenes develop malignant thyroid nodules giving lung metastases (10). This animal model demonstrates an additive affect of the two oncogenes on neoplastic thyroid growth.

In the present study, we searched for mutations of the TShR and Gsα genes in a group of hyperfunctioning and nonfunctioning follicular thyroid adenomas specifically se-

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lected to be homogeneous in their gross anatomy (single nodule in an otherwise normal thyroid gland). The histological features of most of these nodules were undistinguishable, but some presented as scintigraphically "hot" hyperfunctioning nodules, whereas others were scintigraphically "cold" nonfunctioning nodules.

Patients, Materials, and Methods

Patients

Included in this study were 30 patients submitted to surgery for a solitary thyroid nodule originating in an otherwise normal gland. Before surgery, 15 patients were diagnosed as having a hyperfunctioning (toxic) thyroid adenoma (a hot nodule at ^{131}I scintiscan with suppression of the extranodular thyroid parenchyma), and 15 patients were diagnosed as having a nonfunctioning (scintigraphically cold) nodule. Cytological smears by fine-needle aspiration showed a pattern of follicular adenoma (11) in all nodules. Patients with hyperfunctioning thyroid adenomas were thyrotoxic with high serum FT4 and FT3 concentrations and undetectable serum TSh by a sensitive method. All patients with nonfunctioning thyroid nodules were euthyroid. Thyroid autoimmunity was excluded for the absence of circulating thyroglobulin, thyroperoxidase, and TShR antibodies. Patients with hyperfunctioning thyroid adenomas were prepared to surgery with methimazole and iodide. Most patients with a nonfunctioning thyroid nodule had been treated with L-thyroxine at TSh-suppressive doses before surgery. Thyroid volume was calculated by ultrasonography using to the formula of the ellipsoid model (12). Normal reference values for thyroid volume were obtained by measuring thyroid size in 130 healthy adults residing in areas with sufficient iodine intake. Mean ± SD thyroid volume was 11.3 ± 3.4 mL in 65 males and 8.6 ± 2.2 mL in 65 females. Age and sex of patients and findings of thyroid ultrasonography are shown in Tables 1 and 2. Thyroid lobectomy was performed in all patients.

In vitro *tests*

FT4 and FT3 were measured by a RIA after chromatographic separation of the free hormone (FT4 RIA, FT3 RIA, Lysophase; Technogenetics S.r.l., Milan, Italy). TSH was assessed by a sensitive assay (AutoDELFIA hTsh Kit; Pharmacia s.p.a., Milan, Italy). Thyroperoxidase and thyroglobulin antibodies were measured by passive agglutination (SERODIA-AMC and SERODIA-ATG; Fujirebio, Tokyo, Japan). TShR antibodies were searched for using a commercial radioreceptor assay (TRAK assay; B.R.A.H.M.S., Berlin, Germany).

TABLE 1. Age, sex, and findings at thyroid ultrasound in patients with hyperfunctioning nodules

Patient no.	Age	Sex	Nodule		Contralateral lobe
			Volume (mL)[a]	Ultrasound pattern[b]	Volume (mL)[a]
1	46	F	83	S, H	3
2	51	F	46	M	1
3	46	F	140	M	2
4	49	M	74	M	3
5	29	F	61	M	3
6	51	M	23	S, H	2
7	61	M	26	S, I	4
8	36	F	9	S, I	3
9	41	F	26	M	3
10	40	F	52	S, I	2
11	36	M	74	M	3
12	37	F	10	S, I	3
13	36	F	27	S, I	3
14	66	F	17	S, I	5
15	30	F	6	S, I	4

[a] Volume of nodules and of contralateral lobes were calculated according to the ellipsoid formula (see *Patients, Materials, and Methods*).
[b] S, solid; M, mixed; H, hypoechogenic; I, isoechogenic.

TABLE 2. Age, sex, and findings at thyroid ultrasound in patients with nonfunctioning nodules

Patient no.	Age	Sex	Nodule		Contralateral lobe
			Volume (mL)[a]	Ultrasound pattern[b]	Volume (mL)[a]
1	49	F	13	S, H	2
2	57	M	67	S, I	3
3	51	F	2	S, I	3
4	50	F	32	S, I	3
5	18	M	45	S, I	4
6	30	M	18	S, H	2
7	25	F	7	S, H	4
8	22	F	18	M	3
9	27	F	12	M	6
10	36	F	3	S, I	2
11	32	M	7	S, I	3
12	33	F	7	S, H	1
13	44	F	5	S, I	3
14	37	M	4	S, H	5
15	20	F	29	S, I	2

[a] Volume of nodules and of contralateral lobes were calculated according to the ellipsoid formula (see *Patients, Materials, and Methods*).
[b] S, solid; M, mixed; H, hypoechogenic; I, isoechogenic.

Sequence determination

Genomic DNA was extracted from hyperfunctioning and nonfunctioning thyroid nodules and from the normal extranodular tissue when available, as described previously (13). Direct sequencing of exons 9 and 10 of the TShR and of codon 201 and 227 of the Gsα gene were performed using previously described methods (13). At least two different PCR amplifications from genomic DNA were sequenced on both strands with sense and antisense primers.

No other coding region of the two genes were sequenced. All the mutations identified were subcloned in a plasmid, and sequences were repeated on individual clones. Contamination problems were ruled out by including PCR control samples with no DNA as template. Extraction of DNA and pre-PCR reactions were performed in different rooms with respect to post-PCR reactions.

Results

Histology

Each thyroid nodule was examined by two pathologists (P. Via and AGM), who were blinded for the clinical diagnosis. The histological diagnosis was always concordant. All thyroid nodules were circumscribed by a complete fibrous capsule. The capsule was thin in all hyperfunctioning adenomas, whereas 5 of 15 nonfunctioning nodules showed a thick fibrous capsule. All nodules showed a prevalent microfollicular architecture, but macrofollicular areas of variable sizes were also observed. All hyperfunctioning nodules and 13 of 15 nonfunctioning nodules were diagnosed as follicular adenomas. Two nonfunctioning thyroid nodules showed features of malignancy. One nodule was a minimally invasive follicular carcinoma, and the other nodule was a micromacrofollicular nodule with a focal area (15 mm) of papillary carcinoma.

Genetic analysis

A TShR gene mutation was found in 12 of 15 hyperfunctioning thyroid adenomas (Table 3). One hyperfunctioning adenoma, which was negative for TShR mutations, showed a mutation in codon 227 of the Gsα gene (Table 3). The

TABLE 3. Base substitutions and amino acid modification in hyperfunctioning nodules

TSh gene codon	Gsα gene codon	Base substitution	Amino acid change	Patient no.
486		ATC/ATG	Ile/Met	6
619		GAT/GGT	Asp/Gly	13
623		GCC/GTC	Ala/Val	9
629		TTG/TTT	Leu/Phe	11, 14
630		ATC/CTC	Ile/Leu	5
632		ACC/ATC	Thr/Ile	4, 7, 10, 15
633		GAC/GAG	Asp/Glu	1, 12
	227	CAG/CAT	Gln/His	3

mutations of the TShR and Gsα genes identified in the present study were heterozygotic and somatic. All TShR and Gsα mutations had been described already in our (13) and other laboratories (5) and had been found to activate constitutively the AC-cAMP cascade after transient expression in COS-7 cells. The I486M mutation of the TShR gene was previously shown to also activate the phospholipase C-diacylglycerol cascade (14). At variance with hyperfunctioning thyroid adenomas, no mutation of the TShR or Gsα genes was detected in nonfunctioning thyroid nodules.

Discussion

In this study, we searched for somatic mutations of the TShR and Gsα genes in thyroid follicular adenomas lacking the ability to trap iodine as shown by 131-I scintiscan. None of these nonfunctioning thyroid nodules, including the two with malignant transformation, showed a TShR or Gsα mutation. At variance, 12 of 15 hyperfunctioning thyroid adenomas harbored an activating TShR mutation, and in another an activating Gsα mutation was found. These results are in agreement with previous studies indicating that gain-of-function mutations of the TShR or Gsα genes are found in most hyperfunctioning follicular thyroid adenomas (5, 13). Other studies reported a much lower frequency of TShR gene mutations, suggesting that intricate aberrations of the many complex growth-controlling mechanisms are implicated in the pathogenesis of these hyperfunctioning nodules (5, 6). In this regard, data of ADP ribosylation of stimulatory and inhibitory G protein α subunit, together with AC activity in a group of toxic adenomas with and without mutations in the TShR or Gsα genes, suggested that a constitutive activation of the AC pathway may not be sufficient to generate these adenomas (7).

Our results also show that TShR or Gsα gene mutations are not present in nonfunctioning follicular adenomas and, conceivably, they are not involved in the generation of these tumors both as an initial and a secondary molecular event.

This study was performed because experimental models in transgenic mice clearly demonstrated the additive effect of two oncogenes, one of which produced a constitutive activation of the AC-cAMP cascade, on the development and growth of malignant thyroid nodules (8–10). Mutations of the TShR or Gsα genes producing thyroid cell growth might also be permissive for the subsequent development of additional mutations (2, 8, 10), which may abolish some thyroid-differentiated functions or even lead to malignant transformation (2, 8, 10). Activation of the oncogene gsp was described in up to 27% of nonfunctioning follicular adenomas, in up to 12% of follicular thyroid carcinomas, and in up to 13% of papillary carcinomas (15, 16). The presence of activating TShR mutations was also reported in three of six differentiated thyroid carcinomas with high basal AC activity and a poor response to the TSh (17). Other studies failed to detect mutations of TShR or Gsα genes in benign and malignant nonfunctioning thyroid tumors (15, 16, 18–20). However, in most of these studies, screening methods such as single-stranded conformational polymorphism or probes were used to search for gene mutations. Single-stranded conformational polymorphism has a low sensitivity (21), and probes can only identify mutations that have already been described (21).

It is important to emphasize that most thyroid nodules included in the present study had similar pathological features, both macroscopically and microscopically. Hyperfunctioning thyroid adenomas (toxic adenomas) exceptionally progress toward cancer (22). In agreement with this notion, the activation of the AC-cAMP cascade, although leading to proliferation and hyperfunction of follicular thyroid cells, does maintain the differentiation of these cells (2). Malignant transformation is observed in nearly 5% of nonfunctioning microfollicular adenomas and in 25% of nonfunctioning embrional adenomas (1, 23). In keeping with these data, 13% of nonfunctioning follicular thyroid adenomas in our series harbored a cancer. These findings support the concept that nonfunctioning thyroid adenomas do not grow because of TShR or Gsα mutations that activate the AC-cAMP pathway, but due to a different cascade that also leads to the loss of the ability to trap iodine. Recently RET-protoncogene-activating rearrangements have been found in 45% of benign thyroid follicular adenomas appearing after therapeutic or accidental exposure of the gland to ionizing radiation (24). The possible involvement of RET rearrangements in nonfunctioning follicular adenomas originating in nonirradiated thyroid glands is a matter of future studies. An alternative explanation for the pathogenesis of nonfunctioning thyroid adenomas would be the outgrowth of clonal expansion of thyrocytes that never had iodine uptake due to the lack of an NaI symporter protein (25). In such a scenario, even a gain-of-function mutation of the TShR would presumably generate a nonfunctioning adenoma. This sequence of events is not supported by our findings because no activating mutations of TShR or Gsα were found in our series of nonfunctioning thyroid adenomas.

In conclusion, our findings suggest a different molecular pathogenetic mechanism in hyperfunctioning and nonfunctioning follicular thyroid adenomas. Activation of the AC-cAMP cascade, which leads to proliferation but maintains differentiation of follicular thyroid cells, occurs in hyperfunctioning thyroid adenomas. Oncogenes other than the TShR and Gsα are probably involved in nonfunctioning follicular adenomas.

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